WHY ARE LC VIALS SHOWING GHOST PEAKS WITH THE NEW GENERATION OF MASS SPECTROMETERS?

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CHAPTER 1
LEACHABLES FROM SILICON SEPTUM: INFUSION ANALYSIS

INTRODUCTION

When using an automated GC-MS or LC-MS system, the final extract of a sample preparation protocol is usually transferred into a 96-well plate or a 2-mL glass vial. Those containers are then sealed with a flexible material (silicone septum) to allow easy puncture and reseal ability. With an on-going demand to develop mass spectrometers capable of reaching low sensitivity levels, extraneous peaks will inevitably be detected at levels that may trigger an out-of-trend (OOT) or out-of specification (OSS) investigation.[1] This situation leads to the need for additional analytical work to identify/quantify the cause and ultimately offer corrective measures. In 2000, LC-MS rapidly became the predominant choice for analytical work, thus displacing the decade long tested LC-UV solution. At first, the transition between LC-UV and LC-MS was perceived as seamless primarily because of similar recording traces between a UV analog trace and an MS in digital full scan (MS) or single ion recording (SIR). In short, no extra peaks were being detected above a threshold that would trigger investigative work. However, as early as 2005, reports of ghost peaks[2] with newer generation of mass spectrometers started to show up at levels never seen before. These observations lead to noticeable variations during quantification. In order to keep up with new analytical technology, glass vials manufacturers were encouraged to upgrade their manufacturing workflow and quality control to ensure the final product meets these new demands. As seen in Figure 1, a short 15-minute soak test showed on vial with a significant large mass distribution in the mass-to-charge spectrum, clearly indicating a leaching effect at the final stage of a laborious sample preparation protocol.

The term "extractables" and "leachables", or E&L, refers to compounds that can be extracted under extreme conditions (harsh solvent, high temperature, etc.) and to compounds that can migrate or leach by direct contact under normal conditions.[3] Three major business segments are directly affected by the omnipresence of E&L: Food Safety, Pharmaceuticals, and Packaging Manufacturers. The presence of leachables in the food industry came to public light when the Canadian Ministry of Health banned polycarbonate infant bottles, fearing potential exposure to bisphenol A (BPA). In the pharmaceutical industry, containers are not the only source of leachables; drug products such as formulations, fillers, and suspensions are also potential sources. Since 1999, the US Food and Drug Administration (FDA) provided guidance for protection against extractables and leachables with its "Guidance for Industry: Container Closures Systems for Packaging Human Drugs and Biologics".[4,5] In 2005, the European Agency for the Evaluation of Medical Products (EMEA) issued the "Guideline on Plastic Immediate Packaging Materials".[6]

From a workflow point of view, E&L analytical protocols utilize a wide range of extraction, separation, and detection techniques to meet FDA or EMEA regulations. As seen in Figure 2, a controlled extraction study will select options from various solvents to cover a wide polarity range and use several solid-liquid extraction techniques to produce several extracts for analysis by GC-MS (volatile) and LC-MS (non-volatile).[3,7,8,9,10,11] During the extraction process, contact time, additives, and temperature are key parameters to ensure maximum exposure with the extraction solvent.

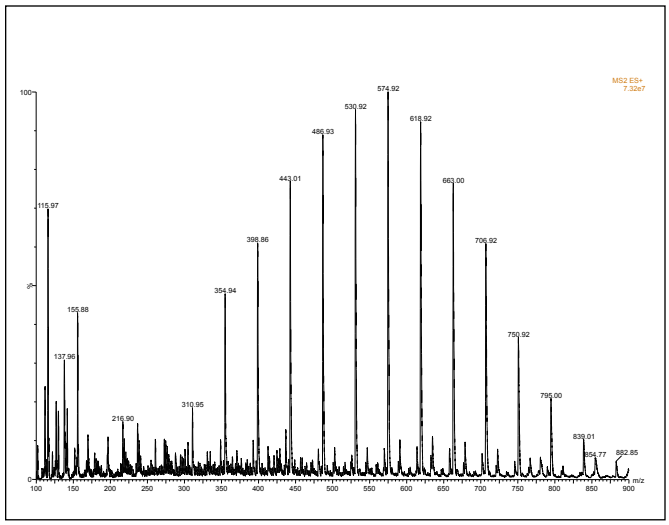

Figure 1. Leachable ion distribution from a silicon cap soaked in methanol.

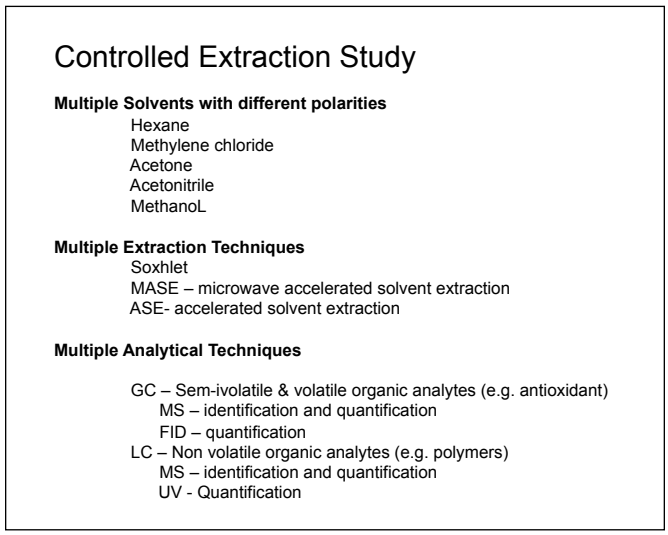

Figure 2. Current extraction protocol and techniques for leachable experiments.

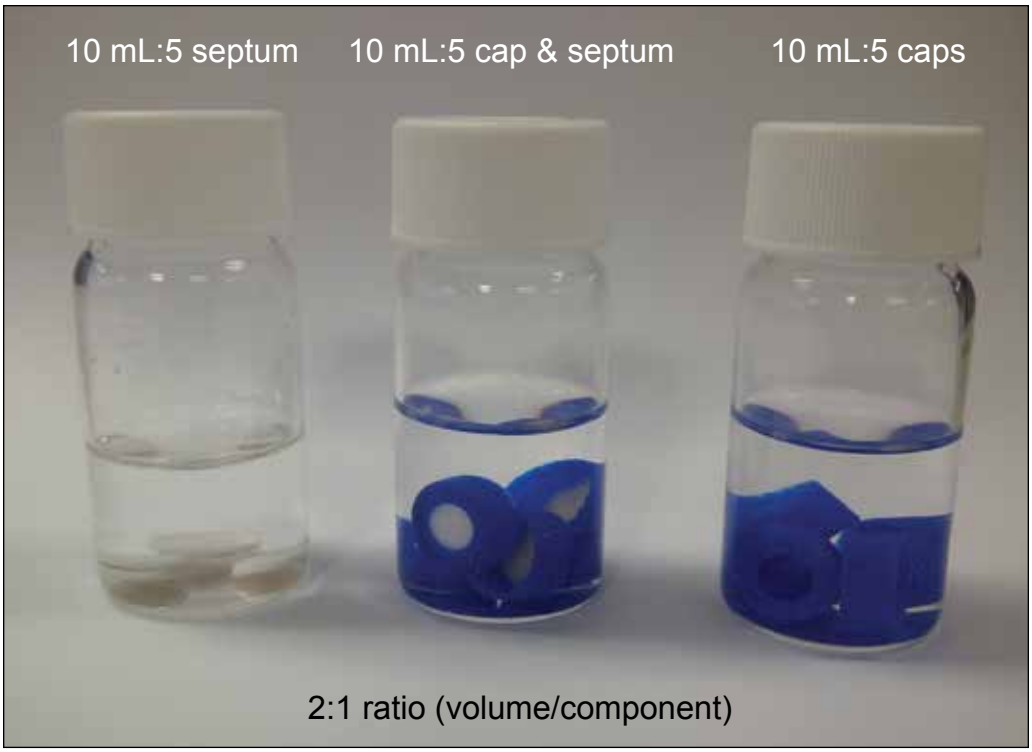

Figure 3. Soak experiment for silicon septum and caps.

EXTRACTION PROTOCOLS

With regard to solid sample extraction techniques, several choices are available, from the century-old Soxhlet extractor, to heated reflux, as well as the time-saving accelerated solvent extraction (ASE) using elevated temperature and pressure (similar to Soxhlet principle). Recently, a relatively new technique utilizing electromagnetic waves as a heat source has been introduced – the microwave accelerated solvent extraction (MASE), which uses closed-extraction vessels and claims fastest extraction times. All techniques are designed to accommodate sample size from small (1 g) to large scale (1000 g). The choice of solvent for extraction will dictate which analytical technique will be used for final analysis.

Currently, liquid and gas chromatography coupled with mass spectrometry are the top analytical choices for analysis. Since extraction techniques can use various solvents to cover a wide polarity range, polar extracts are directed toward LC-MS analysis, and non-polar extracts are typically analyzed by GC-MS. With single-dimension chromatography (LC or GC), the final solvent composition is crucial to ensure proper sample focusing during the injection process. This requirement will produce Gaussian peak shape ideal for qualification/quantitation analysis. However, to achieve acceptable quantification performance at trace level

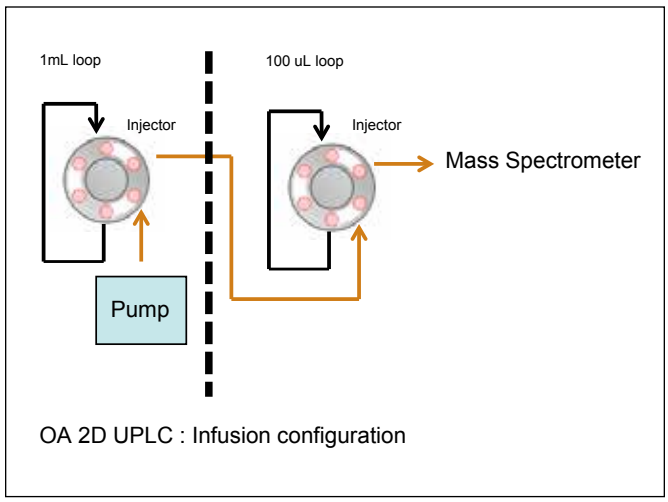

Figure 4. Open Architecture 2D-LC in infusion mode.

(sub ppb), the extraction protocol usually includes a large sample enrichment process. Since current LC-MS and GC-MS are still limited to small injection volumes for analysis, extraction protocol must include a sample volume reduction and a solvent conversion step at the end of the extraction process.

The evaporation and reconstitution step is usually achieved with rotor-evaporators with reduced pressure or by using the gentle nitrogen gas stream technique. In each case, evaporative loss

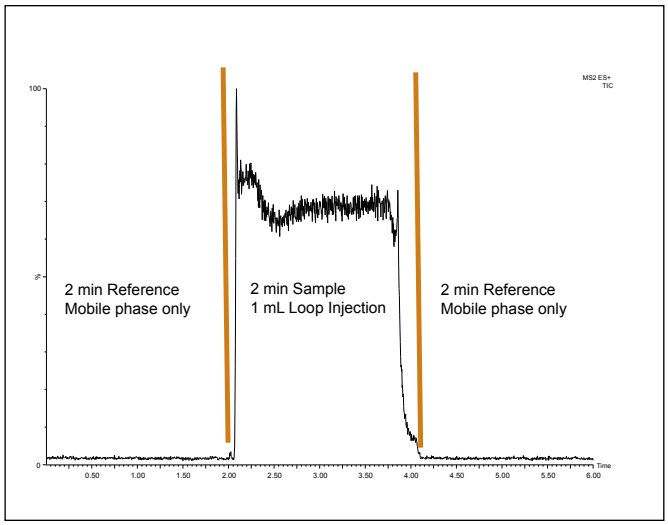

Figure 5. Total ion chromatogram with pre- and post- reference sections.

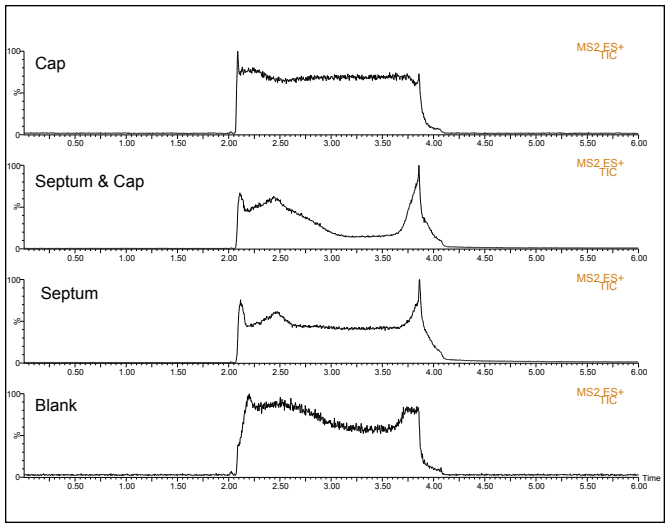

Figure 6. Infusion TICs for septum, cap, and septum/cap profile.

and redissolving issues can occur and cast various levels of uncertainties on the final results. Regardless of the extraction technique, the evaporation/reconstitution step is usually regarded as time-consuming and extremely laborious. With the initial sample volume and final extract volume, an enrichment ratio can be factored during quantification. Since small injection volumes (ex: ≥10 µL LC/MS and ≥1 µL GC/MS) are still used for analysis, macro-extraction protocols are quite inefficient. In fact, it means that only 1% of the final extract (typically 1 mL) is used for measurement and, therefore, 99% of the total work used during the extraction process is simply discarded.

EXPERIMENTAL

As stated earlier, leachables refers to entities that can migrate by direct contact under intended conditions. The solvent volume-to-mass ratio is crucial in order to ensure complete sample coverage. A typical leachable experiment can utilize variable mass-to-volume ratio (i.e., 1:1, 1:10, 1:100 or 1:1000). With the larger ratio, the contact solvent will inevitably needed to be evaporated to dryness and reconstituted in an appropriate solvent for further analysis (LC-MS or GC-MS). For potential leachables present in re-sealable silicone cap used with 2-mL glass vial, septums were placed in a clean 20-mL container with four solvents (water, methanol, acetonitrile, and acetone) for a 60-min time period. The volume-to-mass ratio chosen for this experiment was 2:1, which translate to 5 caps in 10 mL total solvent. The containers were sealed and let at room temperature before analysis (see Figure 3). The 20-mL vials with caps were transferred into a large plate holder and no additional handling was performed with the sample.

The infusion analysis was performed using an Open Architecture UPLC® System with 2D-LC Technology set in "infusion mode." As seen in Figure 4, a 1-mL total sample were aspirated from the 20-mL vial and injected into a 1-mL loop. The infusion analysis collects a 2-min reference signal from the loading pump using a 50:50 water:leach solvent with 0.5% formic acid. After the 2-min reference, the injection loop was pulsed into the infusion stream for a full scan acquisition (100 to 1000 amu) under positive electrospray. With a loading flow rate of 0.5 mL/min, the content of the injection loop was flushed completely after 2 min. At the four-minute marker, the injection loop was pulsed into injection mode and the infusion analysis continued with another two minutes reference. As shown in Figure 5, a total ion chromatogram (TIC) shows the pre and post reference section, with target sample in the middle. At this point, 500 spectrums were combined for analysis.

RESULTS

Infusion analysis

The objective of this research was to evaluate the leachables content of pre-slit PTFE/silicone seal cap for 2-mL glass vials. This study was triggered by an increase in reports from users observing sudden appearance of ghost peaks during method

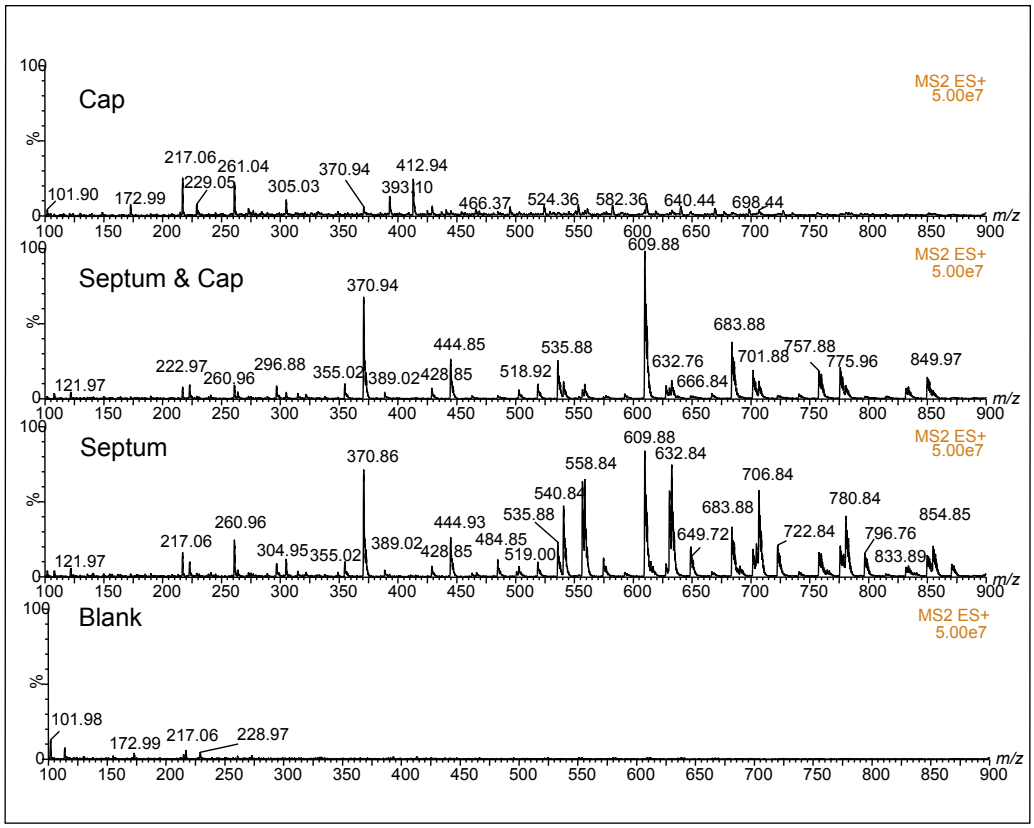

Figure 7. Combined spectrums for septum, cap, and septum/cap.

development and routine analysis. From this point, two multi-dimension chromatography configurations were used to identify leachables present in resealable silicone disks (infusion mode) and to gain insight on chromatography behavior with two-dimensional chromatography with At-Column Dilution mode (2D with ACD mode).

In the infusion mode, a selection of 2-mL caps was picked for leachables study. The re-sealable 2-mL caps are made of a silicone pre-slit material with a Teflon backing acting as an inert barrier. Those silicone caps are currently the preferred format primarily for the option of repeated injections without the need to replace the caps and reduction of evaporative lost effect. Other materials were included in the selection, such as the single injection PE or PTFE caps, and several substitute materials offering flexibility and potential re-sealability. The investigative work started by measuring which part of the cap assembly, the plastic cap or the silicone septum, is prone to leaching. Figures 6 and 7 are showing the TIC and the combined spectrum for: a methanol blank; septum only; septum and cap; and finally, the cap only. The spectrums clearly indicate that the silicone septum is prone to leachable effect, while the plastic cap shows no signal.

The next objective was to determine which solvent has the highest solubility for leachables. In this instance, since these caps are targeted for reversed-phase chromatography applications, water and water-soluble solvents (methanol, acetonitrile, and acetone) were chosen for their compatibility in both sample preparation protocols and chromatography conditions. As seen in Figure 8, the TIC for water shows a weak signal. However, the signals are very strong for methanol (MeOH), acetonitrile (ACN), and acetone (ACE). The combined spectrums show two distinct signal types: a multiple-charge distribution signal with repetitive ions (i.e., 610, 684, 758, 832, etc.); and the single-charge species (i.e., 371). The multiple-charge distribution is a tell-tale signal of polymer entities, which can be de-convoluted for total mass calculation. The ion distribution and intensity between MeOH, ACN, and ACE indicates that leachables components of the silicone septum are highly soluble in a polar solvent. This result suggests that the leachables seen in the MeOH spectrum could have a polar characteristic and potential elute in the early portion of gradient elution with reversed-phase chromatography, thus causing potential matrix effect.

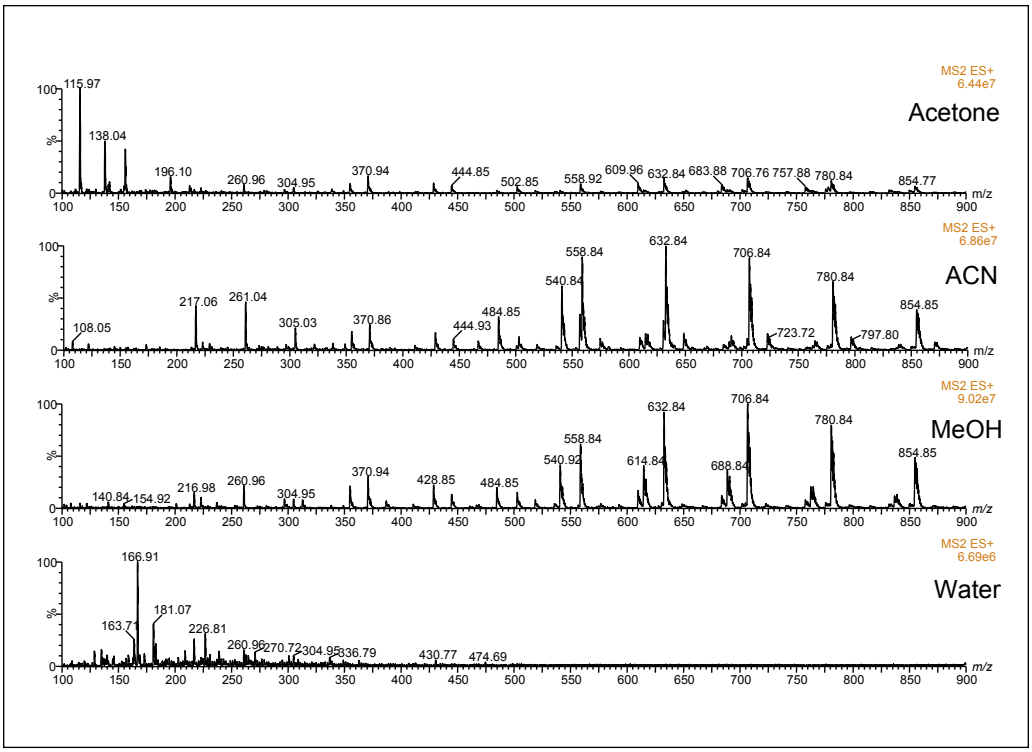

Figure 8. Combined spectrums for water, methanol, acetonitrile, and acetone for silicone septum extract.

The combined spectrums in Figure 9a, 9b, and 9c show the MeOH leachable extracts for 12 2-mL caps; showing results for two single-use only plastic septum, four silicone septum, three competitor silicone septum, and three alternative materials. The results clearly indicate that the PE and PTFE septum show the lowest leachables levels. This result was expected from this type of material under these mild analytical conditions. The single-use cap offers the best performance with respect to leachable levels. Since most applications require replicate injections for reproducibility data, this option has limited applicability. The PTFE/silicon septum remains the industry norm. Proceeding with the analysis, of the seven silicone septum tested, all tested positive for the same 610 multiple-charge series with one exception, which tested negative. This new formulation is the result of an optimized manufacturing procedure in response to high-sensitivity mass spectrometers. Of the three alternate materials, none gave satisfactory results.

CONCLUSIONS

In this application, leachable experiments were conducted with minimum manual labor. The ACQUITY UPLC® System with 2D-LC Technology[12,13] with infusion and at-column dilution configurations enabled 500:1 enrichment analysis by using large-volume injections (aqueous and organic). These two configurations eliminated the time-consuming evaporation-to-dryness and reconstitution steps.

From the results of the experiment, we see there are different levels of extractable masses from different polymers. Some of the polymer materials are not acceptable for MS, as they leach too many masses using solvents common to reversed-phase chromatography.

Within the same septa material (PTFE/silicone) from different suppliers, the levels of leachables using the same solvent conditions yield different results. All manufacturers and suppliers have not achieved the same levels of process and quality controls to offer product clean enough for sensitive MS instruments.

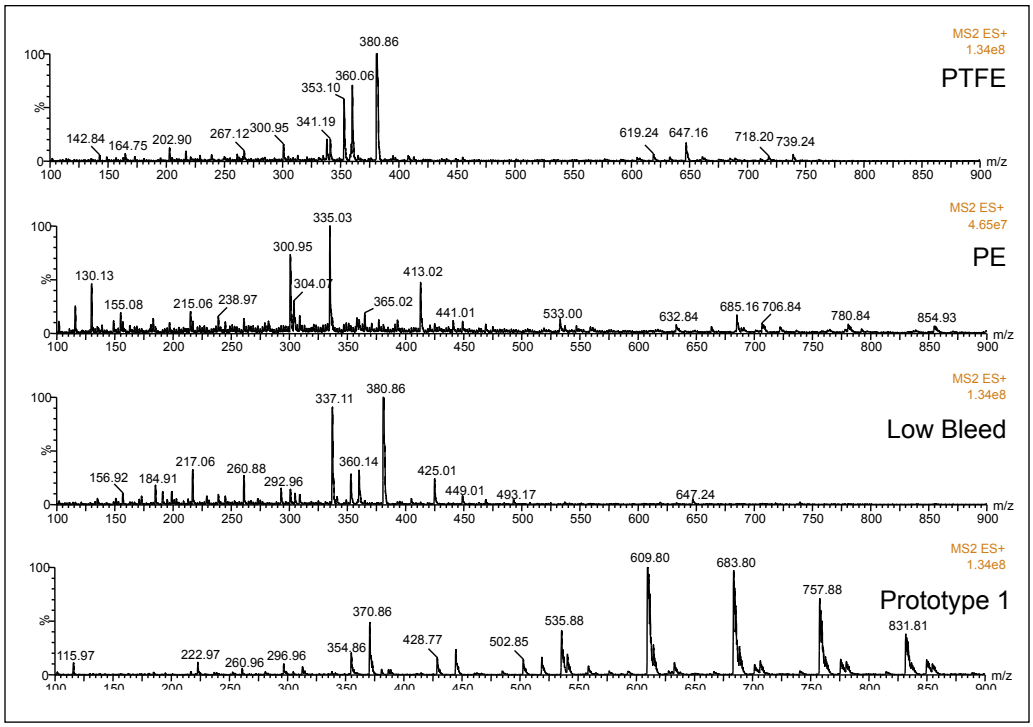

Figure 9a. Combined spectrums (methanol extract) for PTFE, PE, low bleed, and prototype I septums.

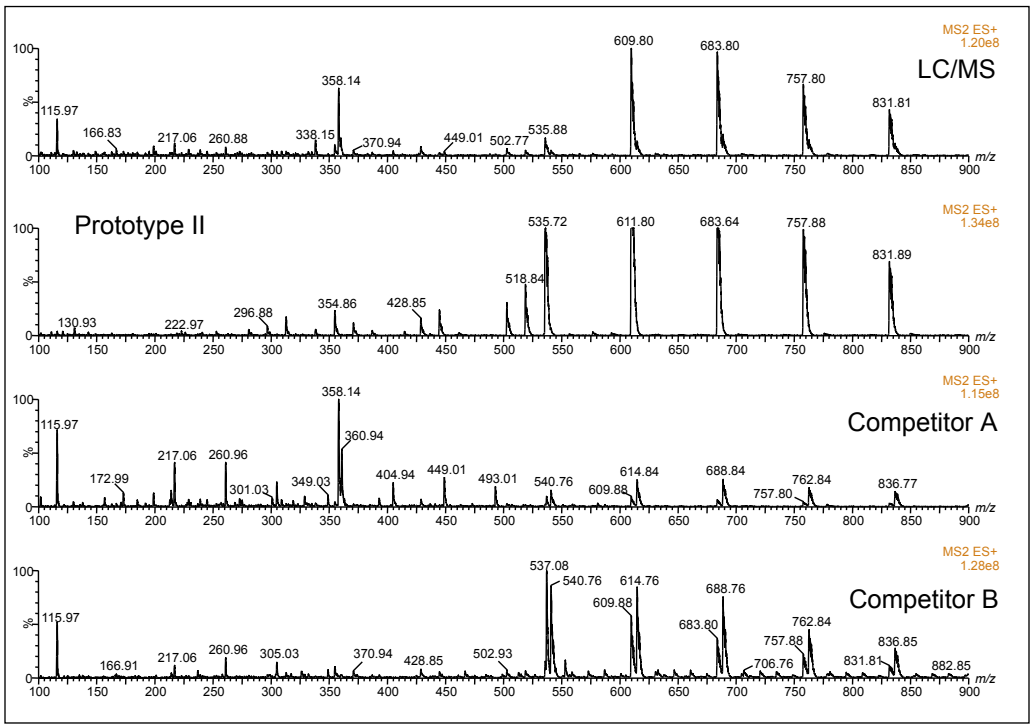

Figure 9b. Combined spectrums (methanol extract) for LC/-MS certified, prototype II, competitor A, and competitor B septums.

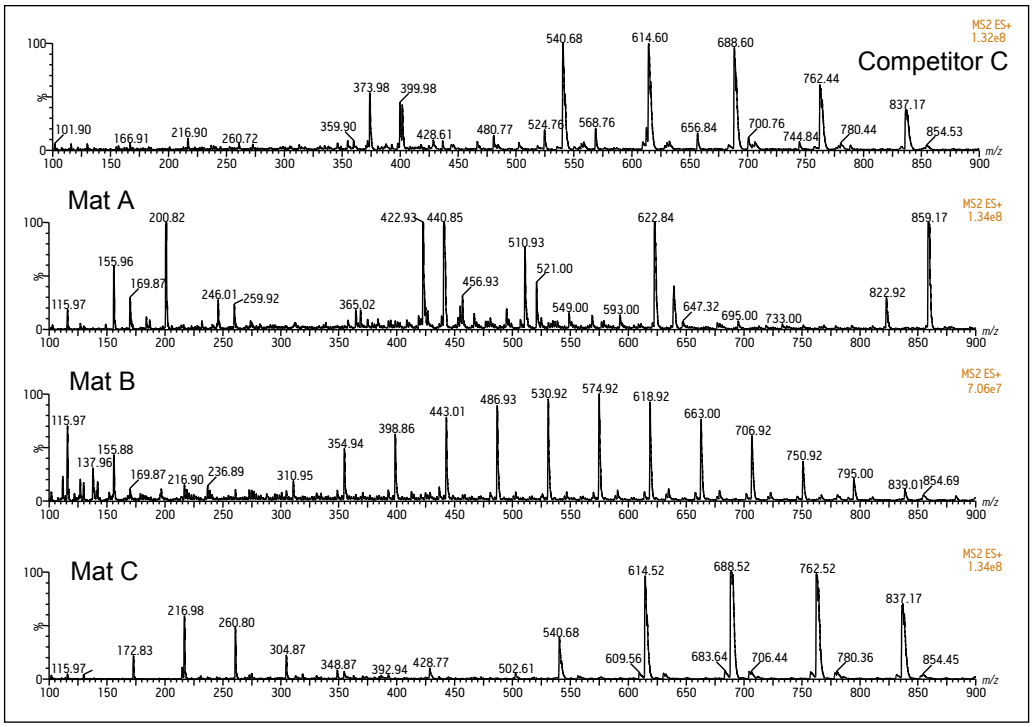

Figure 9c. Combined spectrums (methanol extract) for competitor C, material A, material B, and material C.

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CHAPTER 2
LEACHABLES FROM SILICON SEPTUM: CHROMATOGRAPHY

INTRODUCTION

In this second installation of leachables experiment, we introduce the Open Architecture UPLC System with 2D-LC Technology[1,2] and at-column dilution. This technique demonstrates low-cost enrichment of sample, followed by chromatography, allowing the user to screen a large number of samples in a short time frame.

The example is vials and septa. Packaging material leaching into contents is a common concern in many industries; for example, pharmaceuticals and food, to name two. This technique and instrumentation allows a user to quickly screen materials to select appropriate packaging for the end application.

EXPERIMENTAL

For the enrichment analysis, the Open Architecture UPLC System with 2D-LC Technology was upgraded with the at-column dilution option.[1] The chemistries used for D1 and D1 were the Oasis® HBL 20 µm (2.1 x 30 mm) and the BEH C18 1.7 µm (2.1 x 50 mm) columns, respectively. The loading conditions used for at-column were set at 5% dilution (loader pump at 0.2 mL/min and dilutor pump at 4 mL/min). The injection volume was set at 500 µL for a 4 min loading time.The trapped analytes were back flush eluted with a 0.5 mL/min gradient. The elution started at 5% to 95% organic for 5 minutes with 0.5 % formic acid. Three organic modifiers were used for the chromatography (methanol, acetonitrile, and acetone). The mass spectrometer was set under scan mode (100 to 1000 amu) with positive electrospray (ESI). Each of the 2-mL silicone cap extracts (water, methanol, acetonitrile, and acetone) were subjected to all three chromatography conditions. The 2-mL vial leachable experiments were conducted with the same protocol with one exception. The vials were covered with an aluminum foil to remove the potential contribution of the septum cap.

RESULTS

Given the infusion results presented in Chapter 1, the next step was to evaluate the chromatography behavior. Using the Open Architecture UPLC System with 2D-LC Technology upgraded with at-column dilution, the results for the polyethylene cap, low bleed silicone, and a prototype silicone (acetone extract) are shown in Figure 1a, 1b, 1c, 1d, 1e, and 1f, respectively. In each figure, a total ion chromatogram (TIC), with its corresponding combined spectrum, showcases the chromatography profile for the MeOH, ACN, and Acetone gradient. From each spectrum, an extracted chromatogram was produced for ion with the highest intensity. Also, a second extracted chromatogram was generated for a common ion, in this case the 609.8 from the silicone distribution.

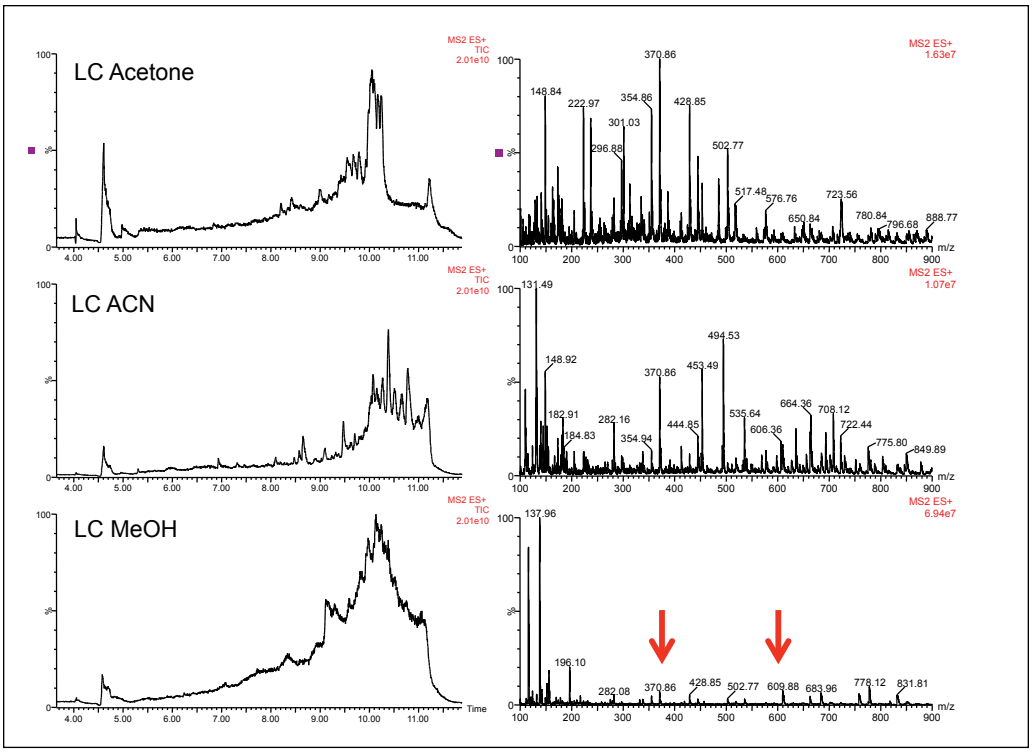

Figure 1a. TIC and combined spectrum for methanol vial extracts.

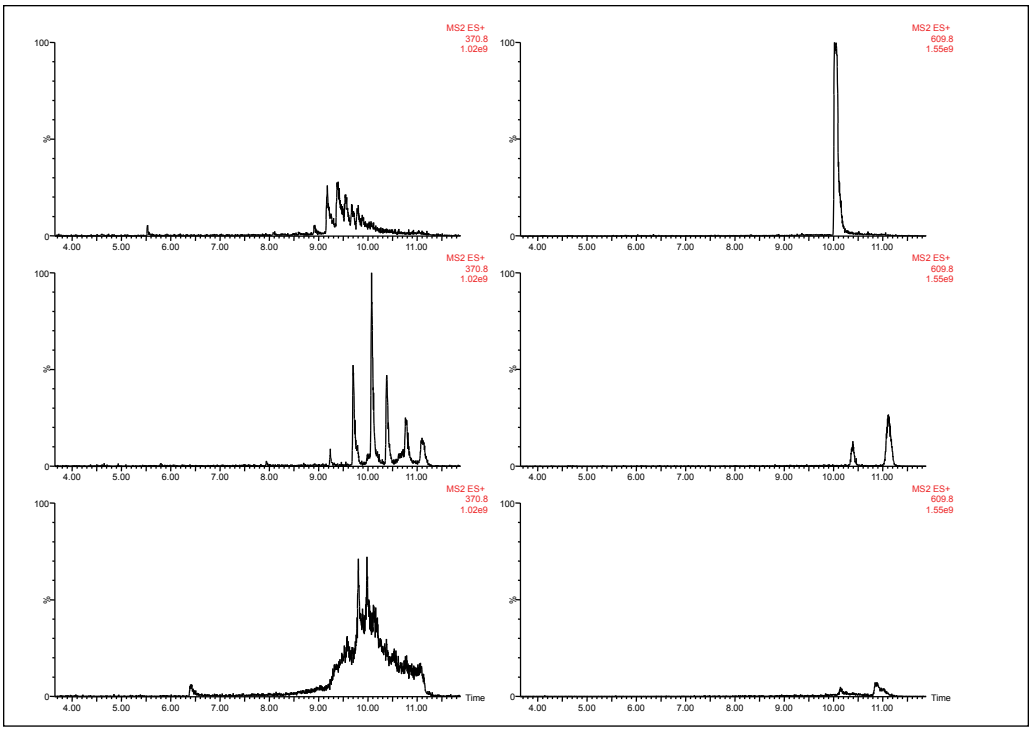

Figure 1b. Extracted mass chromatograms for methanol vial extracts.

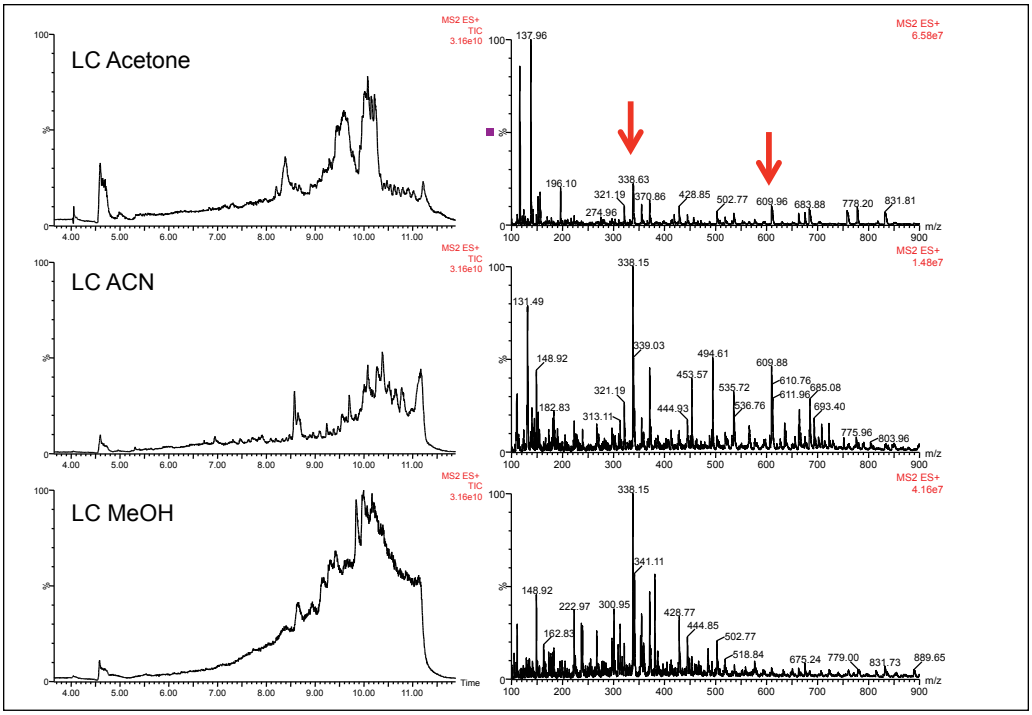

Figure 1c. TIC and combined spectrum for acetonitrile vial extracts.

From the TIC and the extracted chromatograms, it becomes apparent that after the entity's molecular weight is identified, understanding the chromatography behavior is also key for effective identification of potential ion suppression zone during elution. With the polyethylene cap, the 370.8 ion shows an intense signal with an acetone gradient when compared to acetonitrile and methanol. The corresponding extracted chromatogram at 370.8 shows a minor signal with the acetone gradient. However, the 370.8 shows a well resolved distribution with the acetonitrile gradient suggesting that the 370.8 has a high solubility affinity with acetonitrile. The retention time at the end of the gradient suggests a non-polar nature, since the

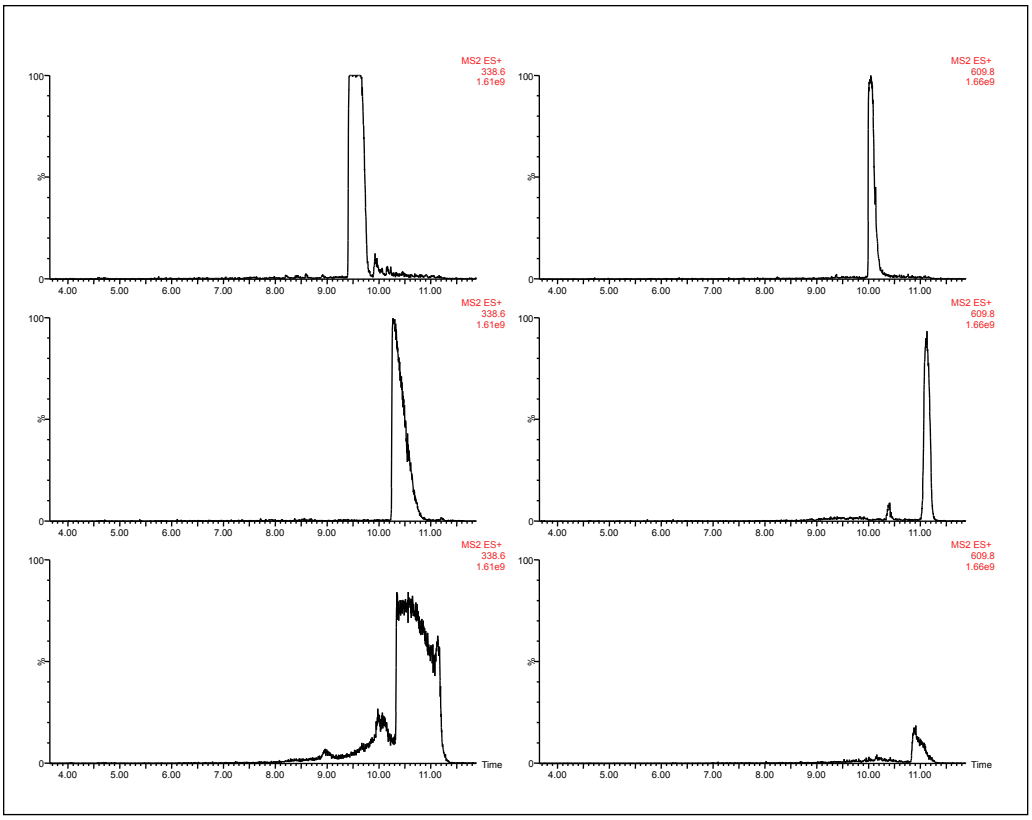

Figure 1d. Extracted mass chromatograms for acetonitrile vial extracts.

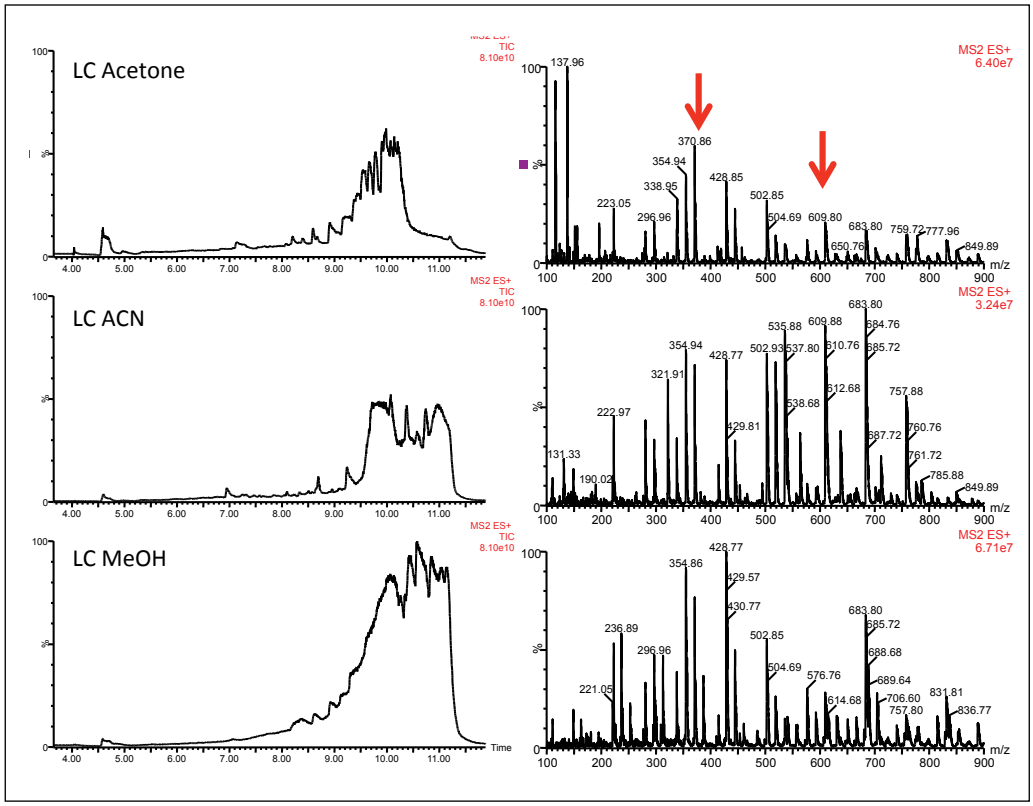

Figure 1e. TIC and combined spectrum for acetone vial extracts.

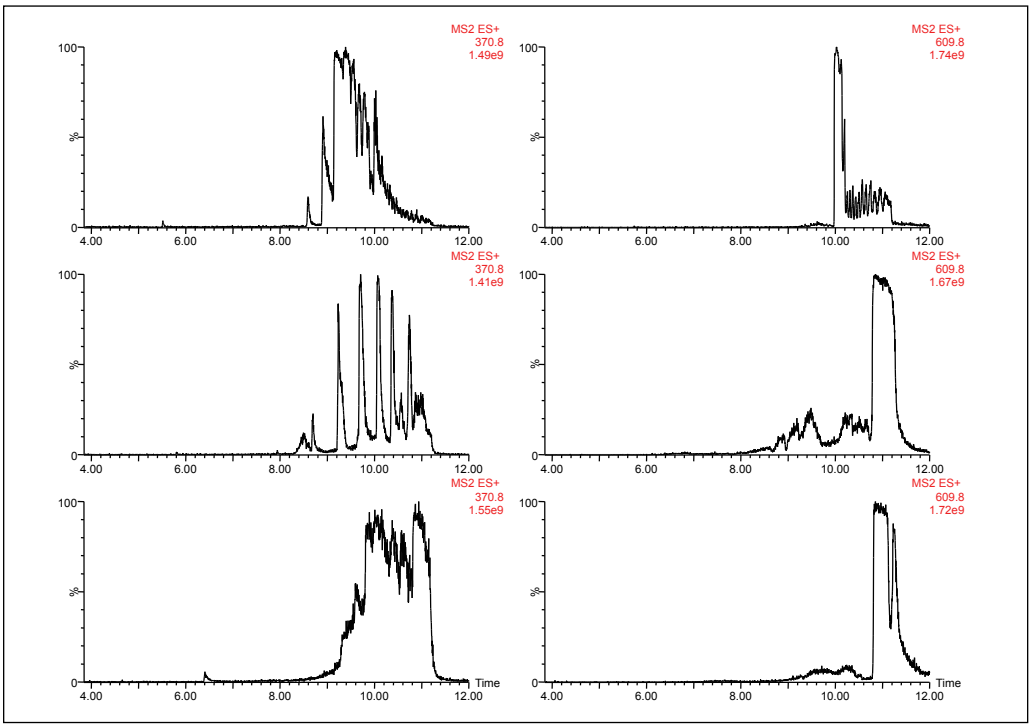

Figure 1f. Extracted mass chromatograms for acetone vial extracts.

separation was performed on a silica-C18 sorbent. The 609.8 ion also shows a late-elution profile, while the high signal and Gaussian distribution with the acetone gradient suggest the 609.8 ion has a stronger affinity for intermediate polarity solvent. The low bleed silicone cap shows a similar distribution for the 609.8 when compared to the polyethylene cap. However, the 338.6 ion shows a chromatography profile with a Gaussian peak shape by using an intermediate polar solvent. As seen in Figure 1c, the methanol gradient produced a distorted signal. However, with acetonitrile, the 338.6 ion produced a sharp rise with a prominent peak tailing, suggesting a better affinity with acetonitrile than methanol. With respect to the prototype silicone cap (see Figure 1f), the chromatography profile for the 370.8 ion shows similarity with the polyethylene cap. The intense signals in all three extracted chromatograms clearly indicate a massive leaching effect with significant matrix effect. The 609.8 ion shows similar result.

CONCLUSIONS

In this application, leachable experiments were conducted with minimum manual labor. The Open Architecture UPLC System with 2D-LC Technology with infusion and at-column dilution configurations enabled 500:1 enrichment analysis by using large-volume injection (aqueous and organic). These two configurations eliminated the time-consuming evaporation-to-dryness and reconstitution steps. Overall, the results indicated a wide range of leachable entities in septum and vials; therefore, trace level analysis (low ppt range) now has to deal with these significant contributions.

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CHAPTER 3
LEACHABLES FROM VIALS: CHROMATOGRAPHY

INTRODUCTION

This is the third installation of leachables experiment using Open Architecture UPLC System with 2D-LC Technology and at-column dilution.[1,2] In this experiment, we further explore the technique by soaking different brands of vials in different soak solvents, then run all extracts in acetonitrile gradients and discuss results. This experiment reinforces the ability to process many samples and solvents in a short timeframe.

EXPERIMENTAL

For the enrichment analysis, the Open Architecture UPLC System with 2D-LC Technology was upgraded with the at-column dilution option1. The chemistries used for D1 and D2 were the Oasis® HBL 20 µm (2.1 x 30 mm) and the BEH C18 1.7 µm (2.1 x 50 mm) columns, respectively. The loading conditions used for at-column were set at 5% dilution (loader pump at 0.2 mL/min and dilutor pump at 4 mL/min). The injection volume was set at 500 µL for a 4-min loading time. The trapped analytes were back flush eluted with a 0.5 mL/min gradient. The elution started at 5% to 95% organic for 5 minutes with 0.5 % formic acid. Three organic modifiers were used for the chromatography (methanol, acetonitrile, and acetone). The mass spectrometer was set under scan mode (100 to 1000 amu) with positive electrospray (ESI). Each 2-mL silicone cap extracts (water, methanol, acetonitrile, and acetone) were subjected to all three chromatography conditions. The 2-mL vials leachable experiments were conducted with the same protocol with one exception: the vials were covered with an aluminum foil to remove the potential contribution of the septum cap.

RESULTS

After a 30-min contact period, five 2-mL vial replicates, from three different vial brands, were individually analyzed for the methanol, acetonitrile, and acetone leaching experiments. The results are presented in Figures 1a, 1b, 1c, 1d, 1e, and 1f, respectively. The chromatograms on the left side are total ion current chromatograms (TIC's) with a 5-min acetonitrile gradient from 5% to 95%, starting at 4 minutes. From time zero to three minutes, each extracts were loaded onto the trap dimension for enrichment. The spectrums on the right are combined spectrums, indicated by the red arrows. The baseline profile is typical of an acetonitrile gradient, with increasing values of the organic modifier. The distinction from one vial to another can be seen from additional well-resolved peaks and also mild to severe baseline distortion. In this case, vial 1 shows a high level of baseline distortion and well-resolved peaks across all three leaching experiments. The methanol leaching shows a higher number of gaussian peaks, while the acetone leaching shows the severe baseline distortion at 6.82 minutes. With vial 2, the methanol leaching shows a milder baseline distortion with no extra peaks, while the acetone leaching shows a comparable profile with vial 1. Vial 3 shows minimum contribution across all

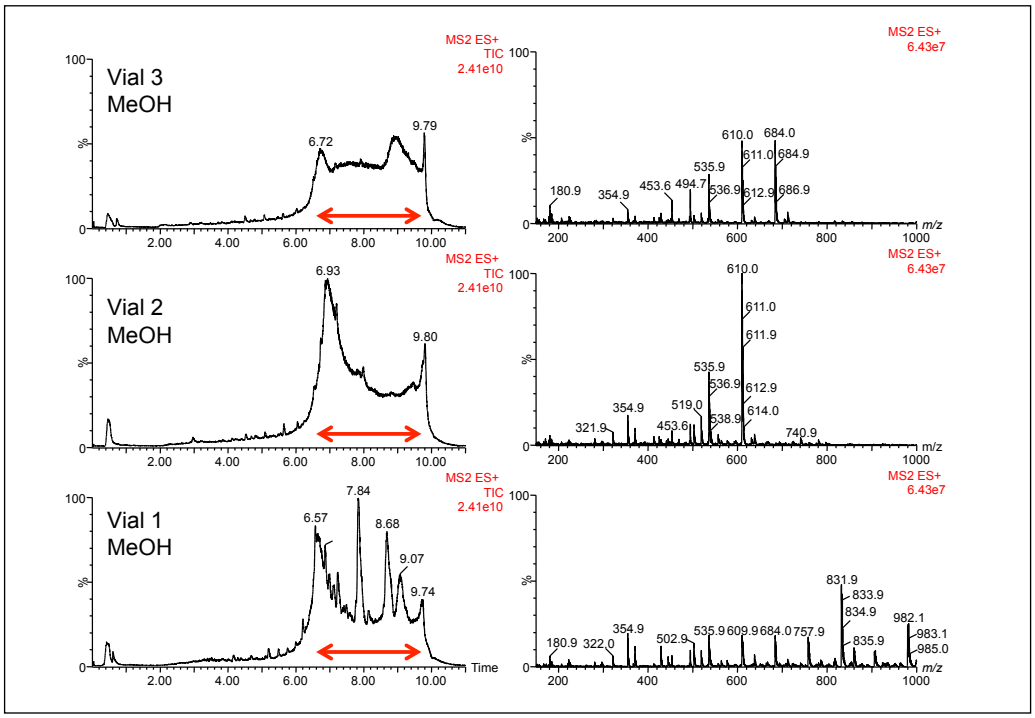


Figure 1a. The chromatograms are total ion current chromatograms (TIC's) in an acetonitrile gradient. Three, 2-mL vial brands soaked in methanol for 30 minutes (5 replicates for each vial brand), covered with aluminum foil and the extract was run on an Open Architecture UPLC System with 2D-LC Technology and at-column dilution. The spectra on the right are the combined spectrum within the red arrows of the chromatogram on the left. The rising baseline is typical for acetonitrile at increasing organic level. Vial 1 shows distinguished Gaussian peaks after 8.5 minutes. At this time, the gradient is high organic showing high reversed-phase retention. Vials 2 and 3 did not show the peaks.

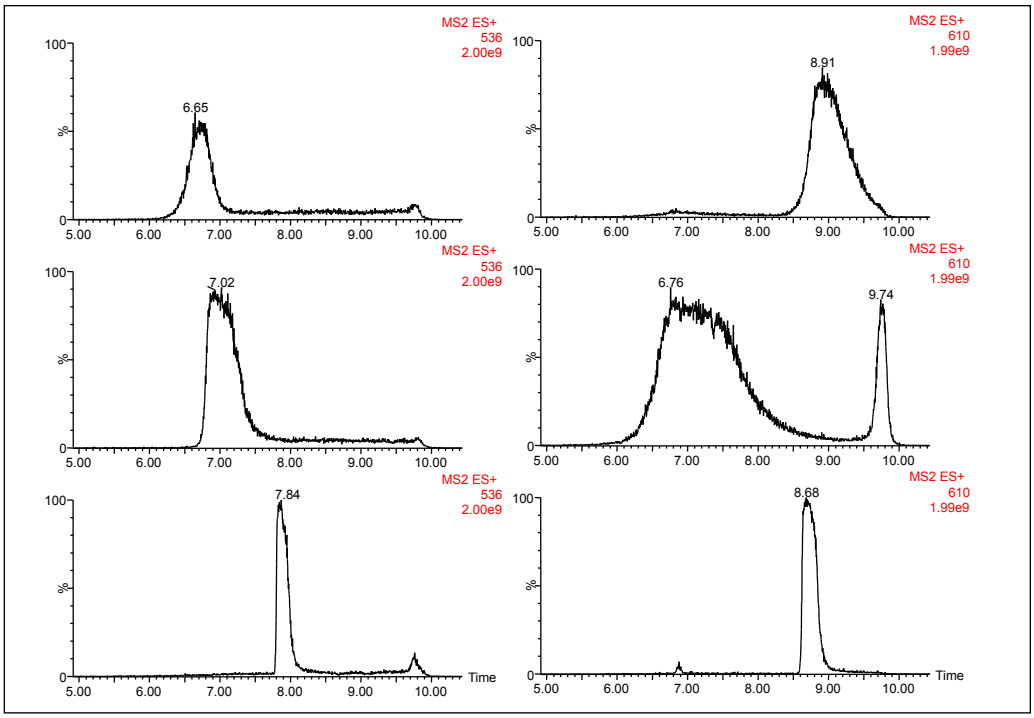


Figure 1b. Two ions were extracted from the TIC, 536 and 610 from figure 1a. 536 ion was well resolved at 7.84 minutes in vial 1; vials 2 and3 have different retention times and wider chromatography profiles. This suggests that the 536 ion, although the same parent mass, could be three different entities.

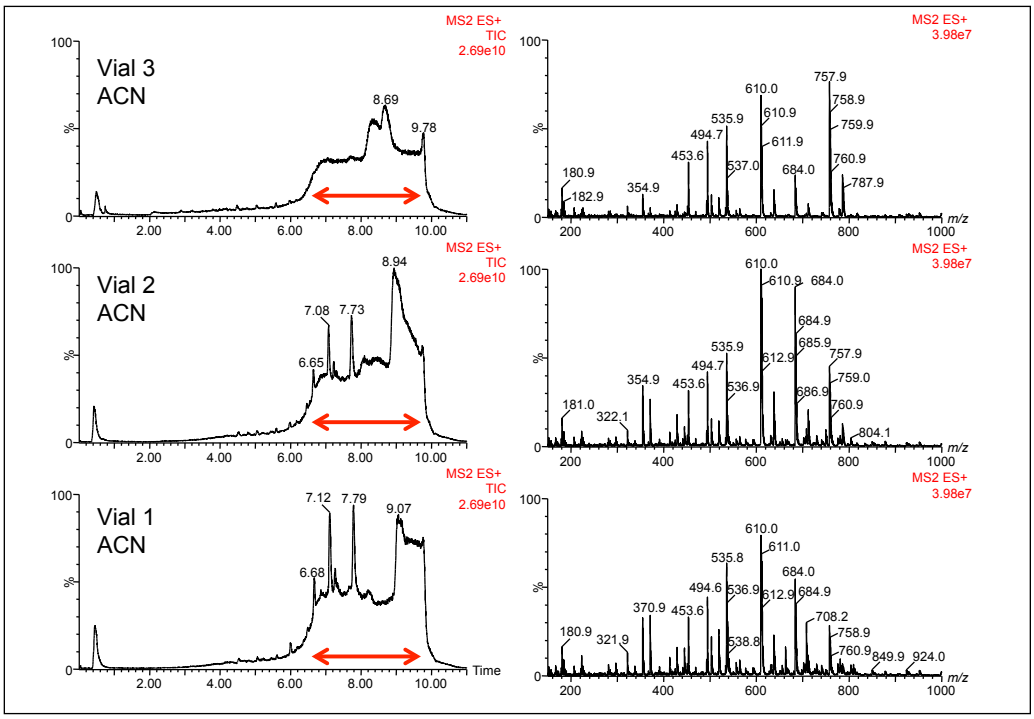


Figure 1c. Three, 2-mL vial brands soaked in acetonitrile for 30 minutes (5 replicates for each vial brand), covered with aluminum foil and the extract was run on an Open Architecture UPLC System with 2D-LC Technology and at-column dilution. The chromatograms on the left were run in an acetonitrile gradient. The spectra on the right are the combined spectrum within the red arrows of the chromatogram on the left.

three leaching experiments, which suggests low leachable entities on the glass surface. From the combined spectrums, extracted mass chromatograms can help visualize the chromatography profile of most abundant ions (*m/z*). For the three vials tested in this experiment, the 536 and 610 ions were extracted from the TIC and presented in Figure 1b (methanol), Figure 1d (acetonitrile), and Figure 1f (acetone). From this data set, the chromatographic behavior of the selected ions can be traced and compared between vial 1, 2, and 3. The 536 ion is seen as a well-resolved peak at 7.84 in the vial 1 methanol extract. However, the same ion is at different retention times and wider chromatography profiles in vial 2 and 3. This could suggest that the 536 ion, although the same parent mass, could be three separate entities. In this case, further MS/MS characterization can add additional information

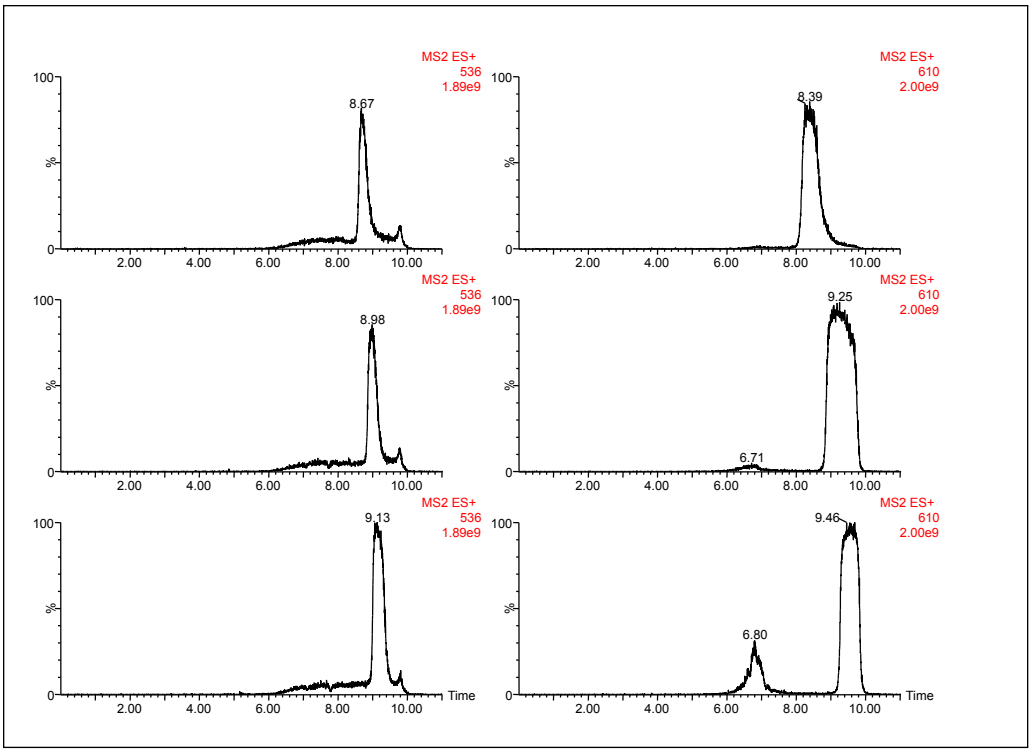


Figure 1d. Low bleed silicone septa with an extracted chromatography 338.6 ion shows a distorted signal in methanol and a sharp tailing peak in acetonitrile suggesting intermediate polarity. The mass at 609.8 shows little signal in methanol suggesting low solubility in more polar solvents and strong signals in acetonitrile and acetone, suggesting an affinity for intermediate solvent strength.

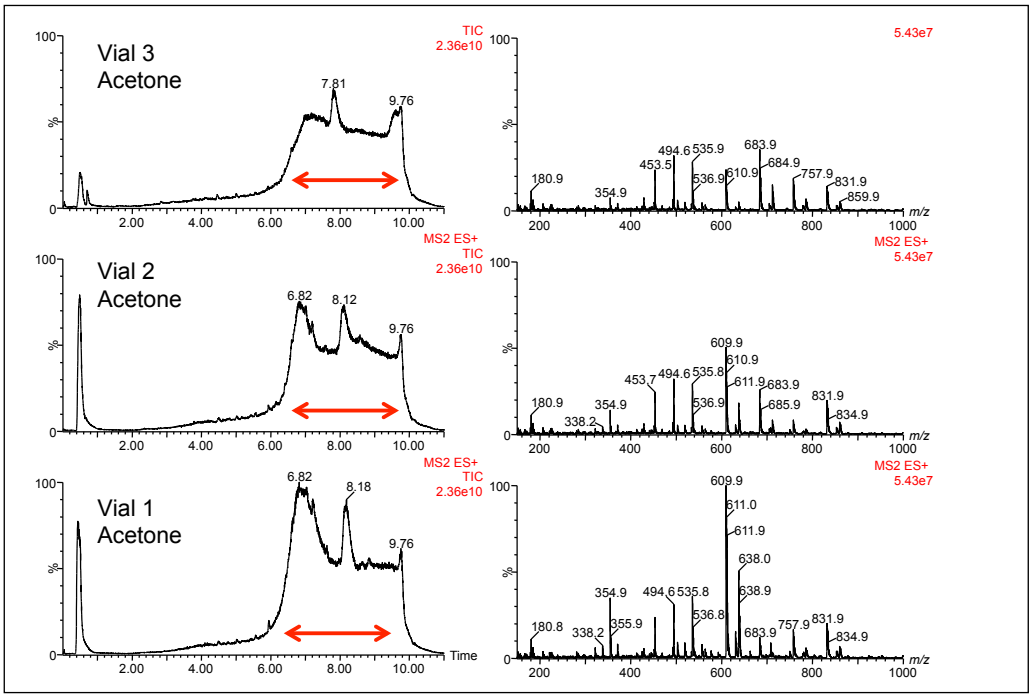


Figure 1e. The chromatograms on the left are total ion current chromatograms (TIC's) in an acetonitrile gradient. Three, 2-mL vial brands soaked in acetone for 30 minutes (5 replicates for each vial brand), covered with aluminum foil and the extract was run on an Open Architecture UPLC System with 2D-LC Technology and at-column dilution. Thespectra on the right are the combined spectrum within the red arrows of the chromatogram on the left. The rising baseline is typical for acetonitrile at increasing organic level.

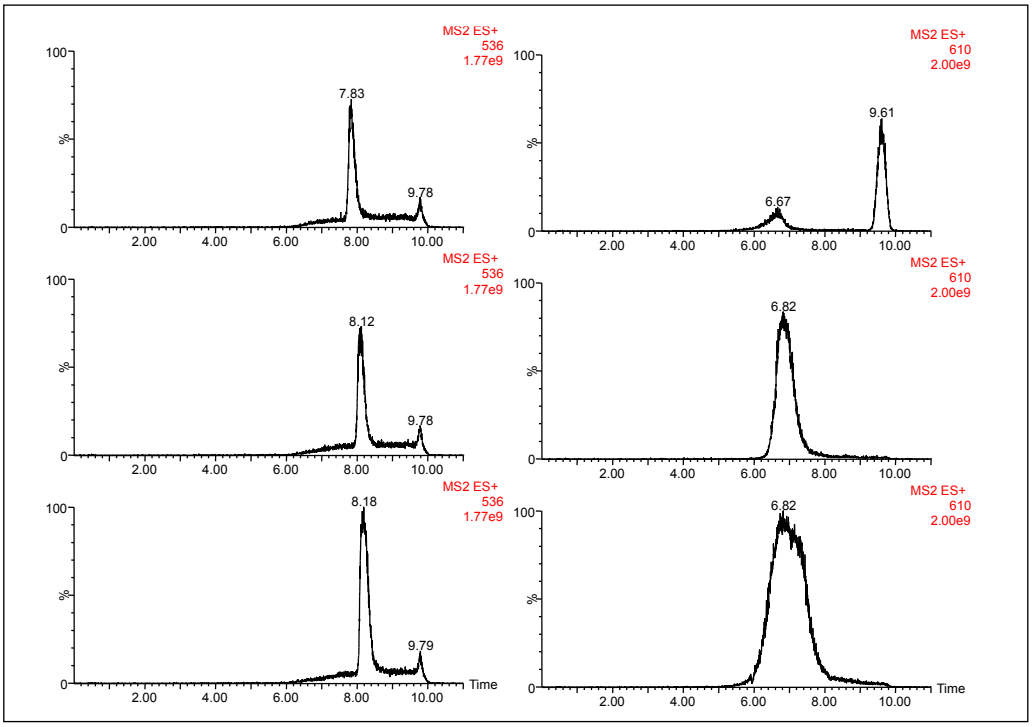


Figure 1f. Two ions were extracted from the TIC, 536 and 610 from figure 1E from the acetone extract. 536 ion shows up later at a similar retention time and a Gaussian peak shape in all three brands of vials. The 610 ion shows also with different retentions in vial 3 than in vials 1 and 2, suggesting vials have same parent mass at 610, but they could be different entities.

with fragmentation experiments. With the acetonitrile extract, the 536 ion shows up at a later and relatively same retention time with a gaussian peak shape in all three vial traces. The same scenario is seen with the acetone extract for all three vials. The 609 ion was selected because of it was previously detected in all silicon septum leaching experiments. The 609 ion is part of a silicon distribution (see Figure 1A). As expected, the 609 shows up as a later eluter and highly soluble in methanol, acetonitrile, and acetone.In this case, further MS/MS characterization can add additional information with fragmentation experiments. With the acetonitrile extract, the 536 ion shows up at a later and relatively same retention time with a gaussian peak shape in all three vial traces. The same scenario is seen with the acetone extract for all three vials. The 609 ion was selected because of it was previously detected in all silicon septum leaching experiments. The 609 ion is part of a silicon distribution (see Figure 1A). As expected, the 609 shows up as a later eluter and highly soluble in methanol, acetonitrile, and acetone.

CONCLUSIONS

The techniques demonstrated in the chapters of this paper can be used to screen for acceptable packaging materials or study process of producing cleaner packaging material. Best materials can be selected to protect the package contents without compromising the quality of the contents. Alternatively, this technique can be used to study and improve the process of producing the packaging. Samples can be taken at different process points and conditions to study and control the blooming and leaching of ions from the materials. This is a cost-effective technique to screen for many solvents and process conditions.

References

1. Mallet, C., Multi-Dimension Chromatography Compendium: Trap and Elute vs AT-column dilution, Waters Corporation, 2014. 720005339en.
2. Mallet, C. Why are LC Vials Showing Ghost Peaks with the New Generation of Mass Spectrometers? Chapter 1 –Leachables from Silicon Septum: Infusion Analysis. Waters Corporation, 2014. 720005335en.

CHAPTER 4
MULTI-DIMENSIONAL CHROMATOGRAPHY COMPENDIUM: TRAP AND ELUTE VS AT-COLUMN DILUTION

TRAP AND ELUTE VS AT-COLUMN DILUTION

Hyphenated systems, or multi-dimensional chromatography, is not a new topic; the first hyphenated system (GC-MS) was introduced in mid 1960s. The major benefit of hyphenated systems is the capability of combining key functions from each dimension, which leads to enhanced performance for solving complex analyses. The analysis of a complex samples is the most perceived usage of hyphenated systems, which stems from its increased peak capacity or separation power. Although the high performance of hyphenated systems provides a clear advantage over single-dimensional solutions, hyphenated solutions have been viewed as academic curiosities and too complex for routine application, and are sometimes perceived as being too difficult to operate from a technical perspective. However, with today's computer technologies, hyphenated systems are now available under full automation control. Custom configurations can be built to provide a cost-effective solution for complex sample preparation protocols.

Hyphenated systems provide a clear advantage over single-dimensional solutions. With full automation control, multi-dimensional solutions can be quickly configured to provide several key benefits for difficult applications. The upgrade of a single-dimensional chromatography unit to a two-dimensional (e.g., trap and elute mode one) is a relatively simple process. As show in Figure 1, with the addition of an extra pump, valve, and trapping column, the trap and elute configuration offers the option to inject large sample volume (up to 1 mL) regardless of the sample solvent composition, thus eliminating the need for solvent exchange. This feature is achieved by using a short column packed with a large particle sorbent for the first dimension.

The trapping column (D1) is not designed to be operated under high chromatographic resolution. From the van Deemter equation, the resolution performance of a chromatography process has an intricate link to the particle diameter of the packing material. From a practical aspect, a column packed with small particle size will produce higher resolution performance (Rs) and separation power (Height Equivalent to Theoretical Plate or HETP) with the consequence of creating higher back pressure.

The trap and elute configuration combines both features, by using two distinct column chemistries, each with their optimum operating conditions. The high-resolution column is positioned on the back end of the configuration and directly connected to a detector. The trap column, via a switching valve, is operated in load or elute mode. In load mode, the trap column is now directly connected to an injection valve driven by a loading pump. Since the trap column is packed with large particles (>10 µm), analytes are captured on the trapping material by using optimized flow rates, chemistry, and additives during the loading phase. The main objectives during this step is to use conditions with the highest k' for maximum peak trapping and minimizing potential breakthrough effects. Once the analytes of interest are trapped on the sorbent, the trap column is re-positioned into the elution stream (backflush elution) for analysis.

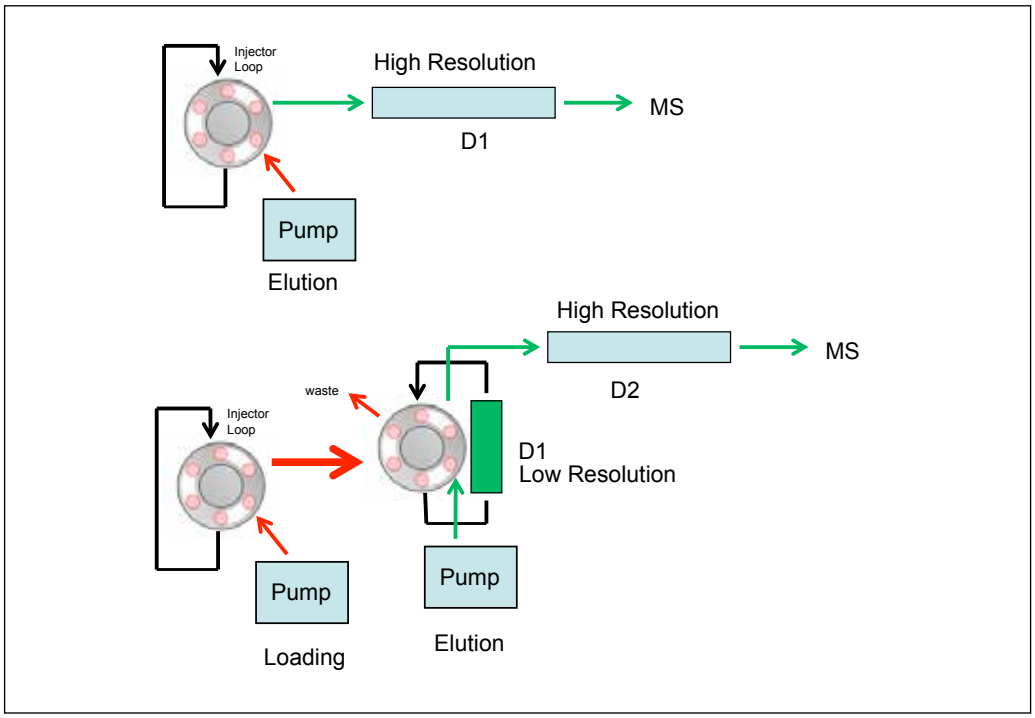


Figure 1. 1D vs 2D configuration with single stream loading.

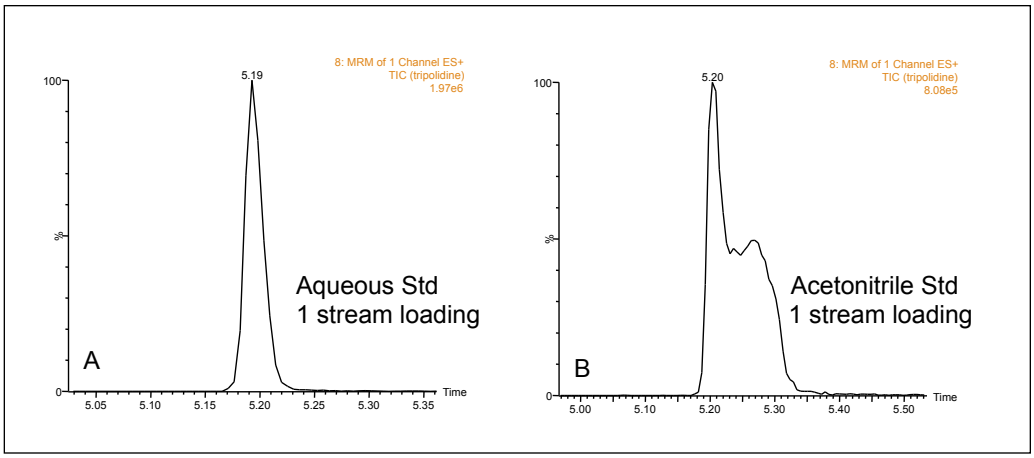


Figure 2. Aqueous vs organic extract using 2D single stream loading.

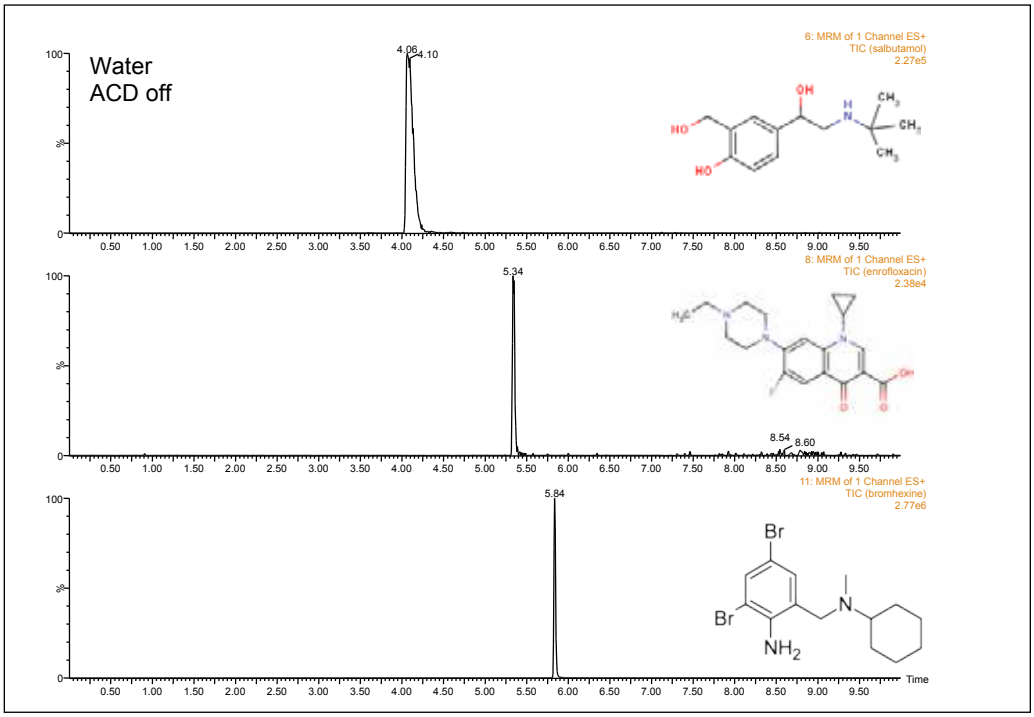


Figure 3. Early-, mid-, and late-eluter in water using 2D single stream loading.

As presented in Figure 2A, an aqueous extract is loaded onto a trap column using a single stream loading. As expected, the profile of the analyte shows a Gaussian distribution. However, the peak is distorted when the same analyte is loaded onto the trap column but in this instance the analyte was dissolved in acetonitrile (Figure 2B). As can be seen, the peak shape shows a distorted profile. The peak splitting profile is the most common behavior when loading k' is not optimized properly. Furthermore, a shoulder peak appears and cause an increase peak width. In this instance, the peak distortion occurs during the loading phase on the trapping column. The loading pump moves the content of the injection loop toward the trapping column, and since the loop volume is made up of 100% organic solvent, the analyte's affinity for the mobile phase will have a high value (low k'). Therefore, in these loading conditions, the analyte's transfer rate from the mobile phase to the stationary will create a dual focusing zone during the injection step. In order to obtain Gaussian peak shape, the analyte must be focused into a tight and narrow band at the column's head. The split peak profile seen in Figure 2B suggests that the acetonitrile percentage is still too high during the injection step, and a significant amount of the analyte travels further into the sorbent bed.

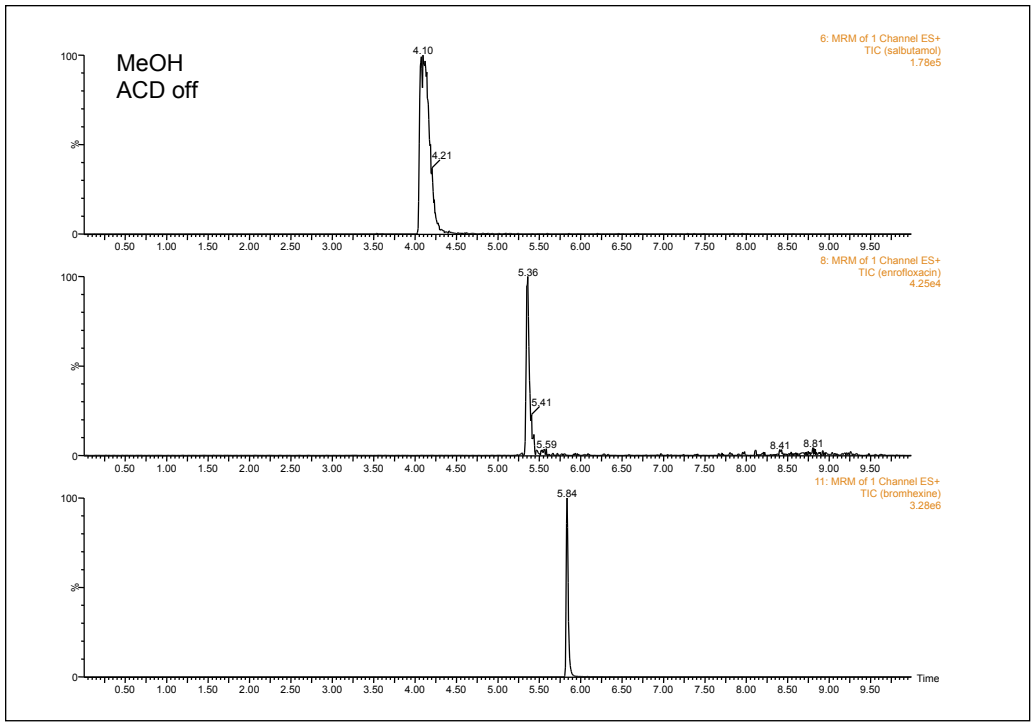


Figure 4. Early-, mid-, and late-eluter in methanol using 2D single stream loading.

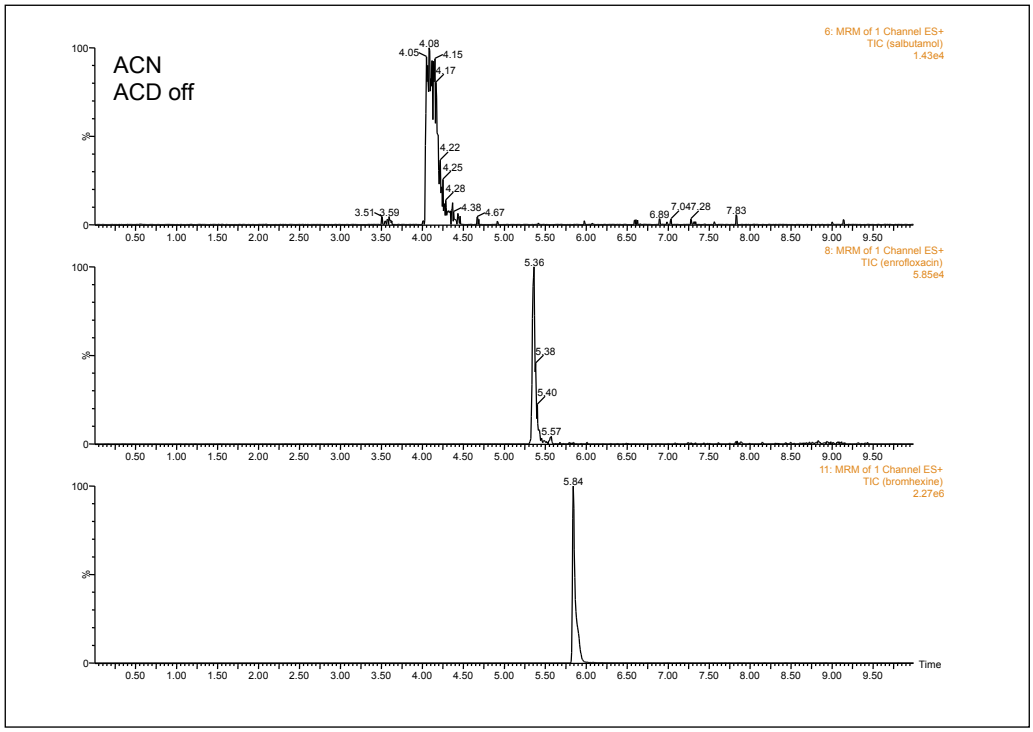


Figure 5. Early-, mid-, and late-eluter in acetonitrile using 2D single stream loading.

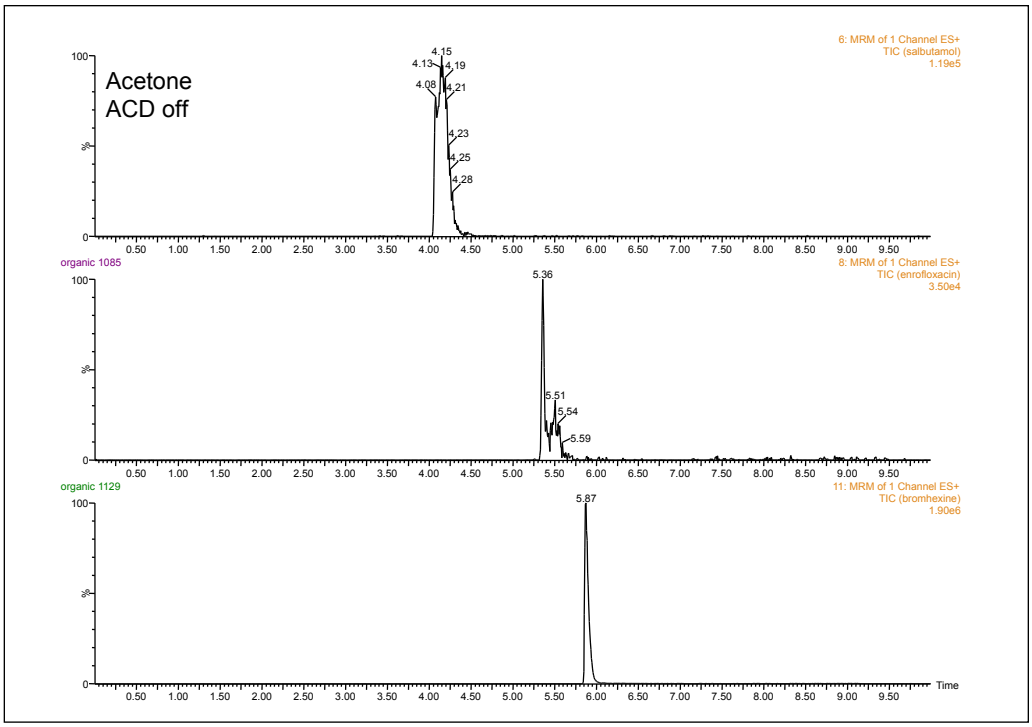
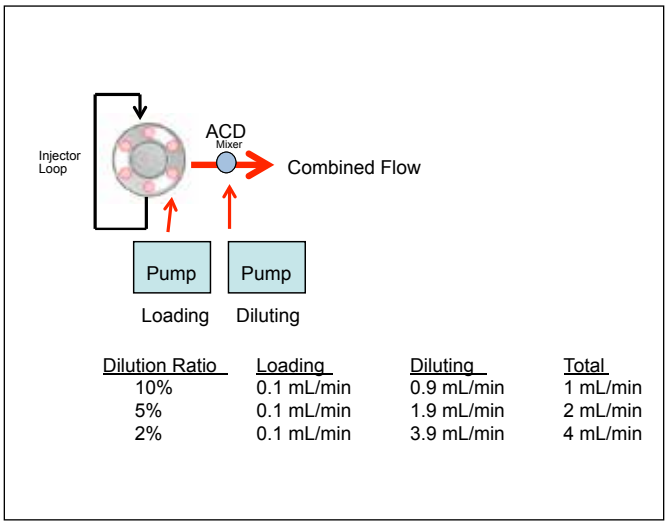


Figure 6. Early, mid, and late eluter in acetone using 2D single stream loading.

Other chromatography profiles are also tell-tale signs of non-optimized loading conditions. The chromatograms in Figure 3 show early-, middle-, and late-eluting peaks with a single stream aqueous loading using an aqueous sample matrix. As it can be seen, all three analytes show excellent Gaussian distribution. However, the early elute shows a wide peak width. This chromatography profile is linked to poor refocusing on the high resolution dimension. In some applications, a target analyte can have a reduced solubility or insoluble in water. In this situation, the loading of an extract dissolved into an aqueous soluble organic will produce peak distortion, as seen in Figures 4 (methanol), 5 (acetonitrile), and 6 (acetone). Since k' is a measurement of an analyte's affinity for a stationary phase and the mobile phase, a target analyte's k' can be affected by its solubility in various organic solvents. As demonstrated for the early-eluting peak, Salbutamol, the analyte shows high e5 signal for the aqueous (Figure 3), methanol (Figure 4), and acetone (Figure 6) results. The chromatogram in Figure 5 with the acetonitrile matrix shows a low e4 signal, suggesting complete breakthrough during loading.

The trap and elute configuration is limited to aqueous extracts only. To achieve effective focusing for organic extracts, the challenge is to reduce the organic percentage by diluting with aqueous (see Figure 7). The dilution is performed by combining two flow streams: one stream is connected to the injector port (loading stream), and the second stream (dilutor stream) is connected after the injector with a low volume mixer (3-way). The flow rates of each stream are set to produce a desired dilution ratio. As shown in Figure 8, two options are available, using a two- or three-pump design. Both designs offer similar analytical performance, however the three pumps approach has a higher dilution ratio capability. The two streams design utilizes a split loading stream to create a dilution effect for the injection volume before reaching the trapping column. This option now offers the possibility to inject both aqueous and organic extract. This configuration effectively utilizes at-column dilution, the benefits of which are shown in Figure 9.



Dilution Ratio	Loading	Diluting	Total
10%	0.1 mL/min	0.9 mL/min	1 mL/min
5%	0.1 mL/min	1.9 mL/min	2 mL/min
2%	0.1 mL/min	3.9 mL/min	4 mL/min

Figure 7. Leachable ion distribution from a silicon cap soaked in methanol.

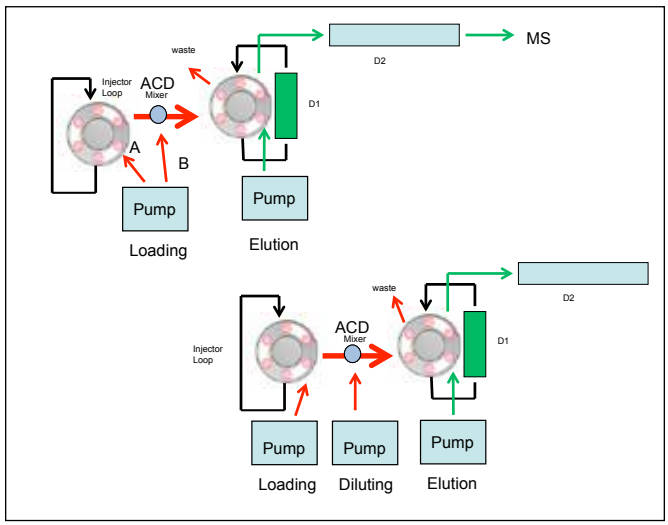


Figure 8. Current extraction protocol and techniques for leachable experiments.

The at-column dilution reduced the amount of organic solvent to an optimum ratio to ensure high k' during peak focusing. As a result, both aqueous and organic injections produce a Gaussian peak shape.

Figure 10 shows the retention profile of three analytes in aqueous (low-, intermediate-, and late-eluting peaks) with the at-column dilution set at 5%. The peak distribution shows a typical Gaussian shape for all three analytes. As expected, since there is no organic solvent in the sample, peaks are showing no tailing or split peak effect. However, the intermediate analyte shows a weak response in comparison to the analytes. In Figure 11, the sample analytes are injected with the same loading and eluting conditions, with one exception being that the sample was dissolved in 100% methanol. As it can be seen, the signal for the intermediate and late-eluting peaks shows increase levels when compared to the aqueous sample (see Figure 10). This can be explained by either a higher solubility in methanol than in water or a reduction of ion exchange retention with the glass vial surface.

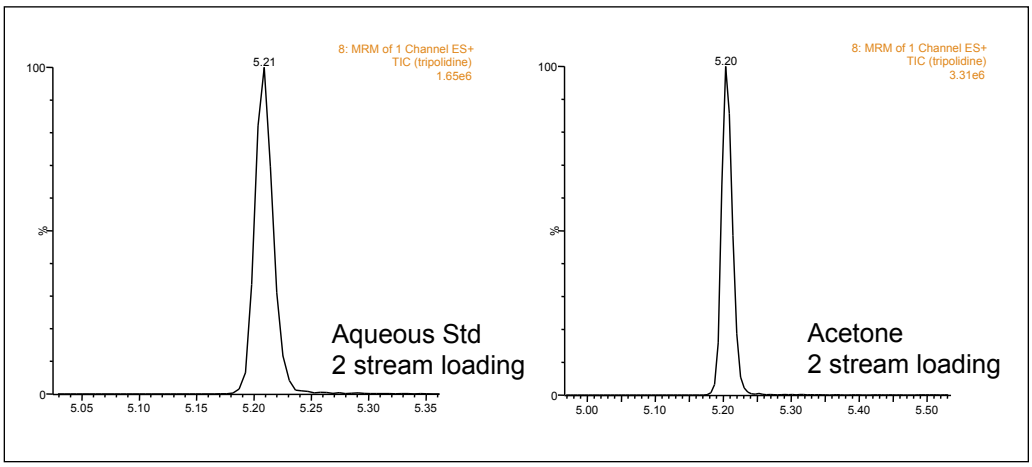


Figure 9. Aqueous vs organic extract using 2D with at-column dilution.

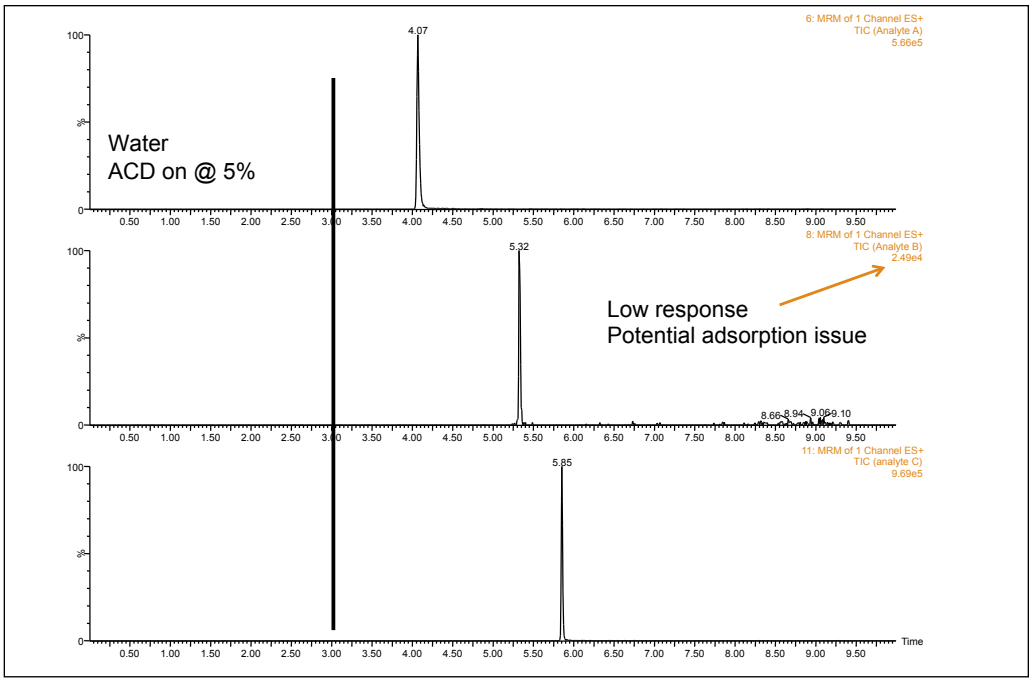


Figure 10. Early-, mid-, and late-eluter in water using 2D with at-column dilution (5%).

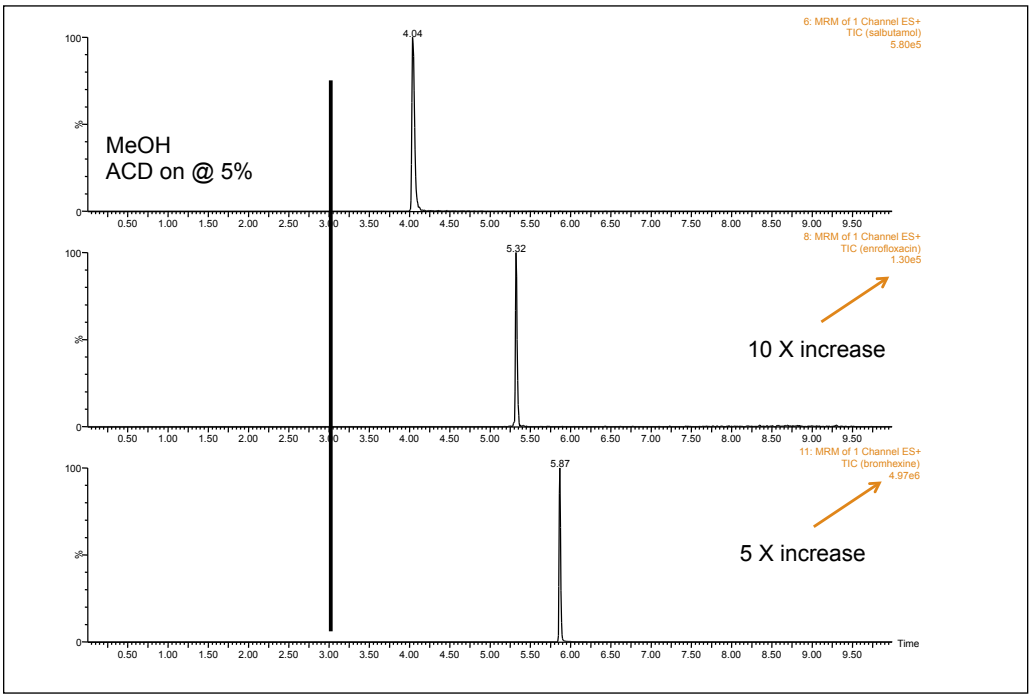


Figure 11. Early-, mid-, and late-eluter in methanol using 2D with at-column dilution (5%).

By decreasing the organic solvent elution strength, Figures 12 and 13, show the result when a sample is dissolved in acetonitrile and acetone, respectively. In these cases, the polarities of the solvents are lower than methanol and can cause peak distortion during peak re-focusing. As it can be seen, the early-eluting peak was drastically affected by a 50% signal drop and the appearance of a shoulder peak. The effect is more predominant if the sample is dissolved in acetone (intermediate polarity) and affects both the early- and intermediate-eluting peaks. The loading conditions require a lower dilution ratio of organic solvent. A three-pump configuration was selected for its high-performance capability (Figure 14) and produced excellent peak shape for the three

analytes in acetone using a 2% at-column dilution factor. The only difference between a 5% and 2% is loading time. As it can be seen, with a 5% loading dilution, the loading time required was set at 3 min for the entire injection loop content to be loaded onto the trapping column. By reducing the loading stream flow rate, the loading time must therefore be extended to achieve optimum focusing on the trapping column.

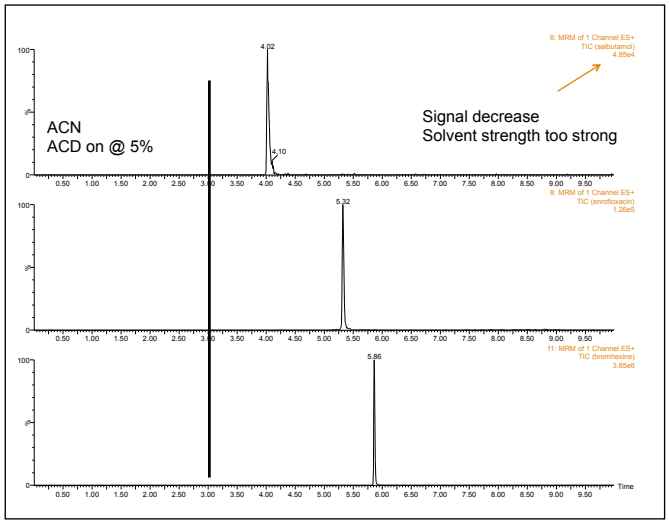


Figure 12. Early-, mid-, and late-elute in acetonitrile using 2D with at-column dilution (5%).

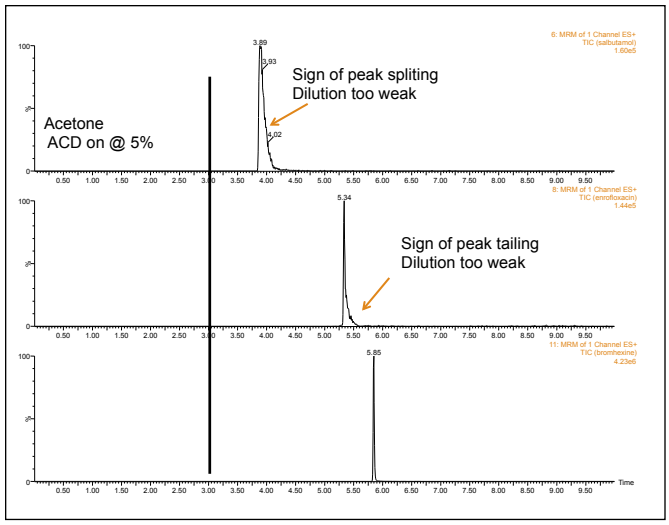


Figure 13. Early-, mid-, and late-eluter in acetone using 2D with at-column dilution (5%).

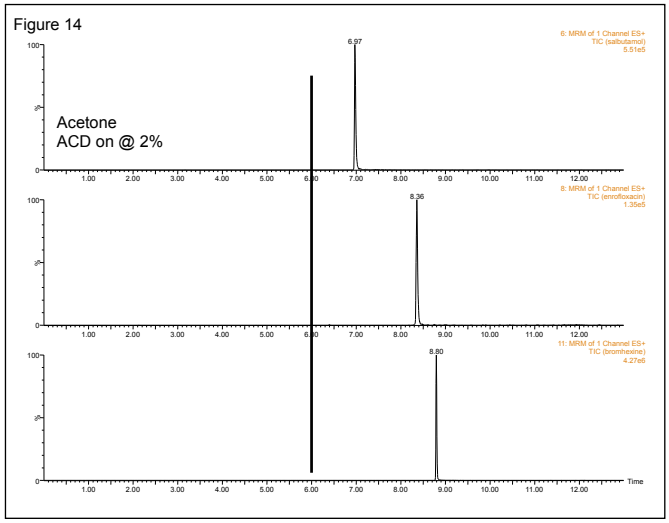


Figure 14. Early-, mid-, and late-eluter in acetone using 2D with at-column dilution (2%).

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